# ASSOCIATION 0F ANGIOTENSIN CONVERTING ENZYME GENE INSERTION /DELETION POLYMORPHISM WITH CORONARY ARTERY DISEASE IN SOUTH INDIAN POPULATION

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# BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **ASSOCIATION OF ANGIOTENSIN CONVERTING ENZYME GENE INSERTION/DELETION POLYMORPHISM WITH CORONARY ARTERY DISEASE IN SOUTH INDIAN POPULATION** is the original bonafide work done by **Dr.A.Leena Devi,** Post Graduate Student Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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# ABBREVIATION

| | | |
|---|---|---|
| AGT | – | Angiotensinogen |
| $AGTR_1$ | – | Angiotensin 11 receptor type 1 |
| $AGTR_2$ | – | Angiotensin 11 receptor 2 |
| CHD | – | Coronary Heart Disease |
| VLDL | – | Very Low Density Lipoprotein |
| LDL | – | Low Density Lipoprotein |
| HDL | – | High Density Lipoprotein |
| ICAM1 | – | Intercellular Cell Adhesion Molecule 1 |
| VCAM1 | – | Vascular Cell Adhesion Molecule 1 |
| PeCAM1 | – | Pericellular Cell Adhesion Molecule1 |
| IL1 | – | Interleukin 1 |
| TNF-α | – | Tumour Necrosis Factor-α |
| PDGF | – | Platelet Derived Growth Factor |
| FGF | – | Fibroblast Growth Factor |
| RAAS | – | Renin Angiotensin Aldosterone System |
| ROS | – | Reactive Oxygen Species |
| eNOS | – | Endothelial Nitric Oxide Synthase |
| DDAH | – | Dimethyl Arginine Dimethyl Amino Hydrolase |
| ADMA | – | Asymmetric Dimethyl Arginine |
| DAG | – | DiAcyl Glycerol |
| PAI1 | – | Plasminogen Activator Inhibitor – 1 |
| LVH | – | Left ventricular hypertrophy |

| | | |
|---|---|---|
| DM | – | Diabetes Mellitus |
| HYT | – | Hypertension |
| SMK | – | Smoking |
| ALC | – | Alcoholism |
| WT | – | Weight |
| HT | – | Height |
| BMI | – | Body Mass Index |
| CHOL | – | Cholesterol |
| TGL | – | Triglyceride |
| EDTA | – | Ethylene Diamine Tetra Acetic Acid |
| DNA | – | Deoxyribonucleic acid |

# INDEX

# INTRODUCTION

Atherosclerosis is the biggest killer of 21st century Mechanisms contributing to atherogenesis are multiple and complex. Multiple theories including the role of dyslipedemia, hypercoagulability, oxidative stress, inflammation, endothelial dysfunction[3,4] have been put forth .Endothelial dysfunction is found to play a key role in the initiation of atherosclerosis .Endothelial dysfunction is characterized by decreased production of vasodilatory Nitric oxide, prostaglandins and increased production vasoconstrictory angiotensin 2 and endothelin1. Increased production of angiotensin 2 also activates free radical injury to endothelium and decreases the availability of NO. This contributes to increased permeability of LDL and its subsequent oxidation in the subendothelial space to oxidized LDL, inflammation and migration of monocytes, macrophages and smooth muscle cells and formation of atherosclerotic lesions .Recently it has been suggested that atherosclerosis continues to have chronic inflammation at every step from the initiation to progression and that all risk factors contribute by enhancing the underlying inflammatory process. Despite changes in lifestyle and the use of new pharmacologic approaches to lower plasma cholesterol[5,6] cardiovascular diseases continues to be the principal cause of death[7, 8]. This led the pathway to the identification of genetic factors that determine the susceptibility to disease .Coronary artery disease (CAD) is a polygenic disease whose phenotypic manifestation depends on the interaction of a number of environmental factors. . The genes encoding components of the

renin-angiotensin system (RAS) present attractive candidates for cardiovascular disease research. The RAS is present in circulating and tissue-based forms and is involved in sodium homeostasis, cardiovascular remodelling, and maintenance of vascular tone. Angiotensin I–converting enzyme (ACE) is a key component within the RAS, where it hydrolyzes angiotensin I to generate angiotensin II (vasoconstrictor) and the kallikrein-kinin system, where it inactivates bradykinin (vasodilator). The observation that ACE inhibitors reduce atherosclerosis in cholesterol-fed rabbits supports the potential role for ACE or its substrates in the development of atherosclerosis.ACE could affect smooth muscle cell and fibroblast migration and proliferation, low-density lipoprotein (LDL) oxidation and endothelial cell function; these are all important factors in atherosclerosis. A polymorphic variant of the ACE gene correlates with higher circulating ACE levels and carries an increased risk of myocardial infarction, and cardiomyopathies. In this study, we sought to determine the distribution of ACE genotypes and the frequency of allele D in patients undergoing coronary angiography at our institution.

# REVIEW OF LITERATURE

Coronary heart disease has been defined as impairment of function of heart due to inadequate blood supply to the heart compared to its needs caused by atherosclerosis. It, being a multifactorial disease has a complex etiology. Many genetic and environmental factors act in combination to determine an individual's risk of developing coronary heart disease[1]. A large number of studies such as the Framingham heart study[2], The Lipid research clinic's coronary primary prevention trial, the Helsinki Heart study, have been conducted to examine the role of risk factors for coronary artery disease. The risk factors identified by these epidemiological studies include a group of fixed risk factors like positive family history, age, male gender, and a group of modifiable risk factors like blood lipid profile abnormalities, hypertension, physical inactivity, obesity, cigarette smoking, alcoholism, diabetes mellitus, hyperhomocysteinemia[2.] Though overwhelming evidence particularly that given by response to "Response to retention hypothesis" indicates that the whole sequence of events is found to be initiated by the retention of modified Low Density Lipoprotein[3,4,]it was identified later that despite changes in lifestyle and the use of new pharmacologic approaches to lower plasma cholesterol[5,6] cardiovascular disease continues to be principal cause of death[7,8] .

## ATHEROSCLEROSIS

Atherosclerosis is a disease affecting arterial blood vessels. It is a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophages, white blood cells promoted by low density (especially small particle) lipoproteins without adequate removal of fats and cholesterol from the macrophages by functional high density lipoproteins (HDL). It is commonly referred to as a "hardening" or "furring" of the arteries. It is caused by the formation of multiple plaques within the arteries.

The lesions of atherosclerosis occur principally within the innermost layer of the artery wall, the intima .They include[9-11],

- Fatty streak
- Fibrous plaque
- Complicated lesions
    - Plaque disruption
    - Atherothrombosis

**FATTY STREAK**

The process of atherogenesis begins in childhood with the development of lipid rich lesions called fatty streaks. They are also found to contain macrophages, T lymphocytes, smooth muscle cells – each of these cells are found to contain deposits of cholesterol and cholesterol oleate. The lesions are yellowish and sessile in appearance and they cause little or no

obstruction of the affected artery and no clinical sequelae. Observations suggest that lipid deposition does not inevitably lead to the advanced lesions of atherosclerosis but a number of factors are associated with the progression of the lesions and with the development of more complex form of atherosclerosis, the fibrous plaque.

**FIBROUS PLAQUE**

The fibrous plaques are derived from fatty streaks that continue the process of cell proliferation, lipid accumulation, and connective tissue formation and that the deep core of lipid and cell debris results from inadequate blood supply, inflammation, and cell necrosis. There is a lesion that is accepted as a forerunner of the fibrous plaque – this is known as fibromusculoelastic or intermediate lesion of the intima, which consists of proliferated smooth muscle cells surrounded by connective tissue and contains little or no lipid. A fully blown fibrous plaque consists of numerous smooth muscle cells surrounded by a dense connective tissue matrix often intermixed with numerous macrophages. This cap covers a deeper layer of macrophages filled with lipid that are often intermixed with variable number of T lymphocytes.

**ADVANCED LESIONS – PLAQUE DISRUPTION AND ATHEROTHROMBOSIS**

The typical advanced, complicated lesion contains a large necrotic core with a fibrous core, loaded with macrophages. The macrophages can form numerous proteolytic enzymes, including metalloproteinases – these enzymes cause the removal of fibrous cap – thus plaque disruption is found

to happen at the shoulder of the lesion where the cap is thin and the concentration of macrophages is the greatest. The plaque disruption allow the lesion to get involved in thrombotic episodes that can lead to occlusive disease[12]Thus, atherosclerosis causes two main problems.

1. First, the atheromatous plaques, though long compensated for by artery enlargement, eventually lead to plaque ruptures and stenosis (narrowing) of the artery and, therefore, an insufficient blood supply to the organ it feeds.
2. Second, if the compensating artery enlargement process is excessive, then a net aneurysm results.

These complications are chronic, slowly progressive and cumulative. Most commonly, soft plaque suddenly ruptures, causing the formation of a thrombus that will rapidly slow or stop blood flow, leading to death of the tissues fed by the artery in approximately 5 minutes. This catastrophic event is called an infarction.

The clinical scenario of this catastrophic event depends on which artery is affected by this event.

1. One of the most common recognized scenarios is thrombosis of a coronary artery, causing myocardial infarction (a heart attack). This condition is called as Coronary artery disease.
2. Second most common is that caused due to thrombosis of carotid artery branches and inadequate blood supply to brain – which presents itself as stroke or transient ischemic attack.
3. Another common scenario in very advanced disease is claudication from insufficient blood supply to the legs, typically due to a combination of

both stenosis and aneurysmal segments narrowed with clots. This is called as peripheral artery disease.

4. Since atherosclerosis is a body-wide process, similar events also occur in the arteries of the intestines, kidneys, legs, etc.

**HYPOTHESIS OF ATHEROGENESIS**

Atherosclerosis has been recognized in humans for thousands of years. Long has discussed the development of clinicopathologic correlations that evolved during the era when autopsy examination permitted the formulation of a hypothesis relating the degree of atherosclerosis to the incidence of myocardial infarction and stroke[13]. Virchow proposed the idea that some form of injury to the arterial wall associated with the inflammatory response resulted in the degenerative lesion of atherosclerosis[14]. This idea was subsequently modified by Antischkow[15] and further included the role of platelets and thrombogenesis in atherosclerosis as expanded by Duguid[16]. John French noted that the structural integrity of the endothelial lining[17] of the artery represented a key element in the maintenance of normal arterial function and that alteration in endothelial integrity might precede a sequence of events that would lead to the various forms of the lesions of atherosclerosis.(FIG ;1)

**RESPONSE TO INJURY HYPOTHESIS**

This hypothesis states that the endothelium helps to regulate the homeostasis of the cardiovascular system. .The endothelium forms the interface between tissues and blood compartment and helps to regulate the entry of molecules and cells in to the tissues in a selective manner .The

ability of endothelial cells to serve as a permselective barrier fails in many vascular disorders including atherosclerosis. The intact endothelium is capable of releasing antithrombotic and fibrinolytic factors in addition to the potent vasodilator nitric oxide (NO). In normal blood vessels, NO and acetyl choline induce vasodilatation, but with endothelial damage,disruption of the cell state negates normal function and the actions of potent vasodilators, the damaged endothelium causes abnormal responses from acetylcholine increasing the production of vasoconstricting agents such as thromboxane A2 and prostaglandins,in addition to eliciting the development of abnormal intracellular ca2+ and endothelin derived vasoconstricting factor

The endothelial dysfunction is associated with overexpression of E, L, P selectin that appear to play a role in inducing rolling and attachment of monocytes and T lymphocytes to endothelium. This rolling is facilitated by the upregulation of ICAM 1 and VCAM 1 also. Another molecule formed by endothelium, PeCAM 1 has been shown to participate in interendothelial migration by the adherent leukocyte into the subendothelial space or intima of the artery. Thus, the earliest phase of the chronic, inflammatory response that has become recognized to be the hallmark of atherogenesis is represented by leukocyte adhesion due to the formation of these attachment and adherence molecules on the surfaces of the endothelium and the leukocytes[18-–20].

A second event accompanying endothelial dysfunction is transmigration of lipoproteins particularly of small LDL particles, this transmigration places LDL in the subendothelial space which is virtually

devoid of any antioxidant properties of the circulation, hence it gets oxidised. Oxidised LDL can act as one of the chemotactic reagents and can also induce the endothelial cells and the underlying smooth muscle cells to form a second chemotactic factor, monocyte Chemoattractant protein 1 (MCP-1) and a colony stimulating factor (M-CSF). In this scenario, the monocyte gets activated to macrophages, which express SR-B1 causing unregulated uptake of LDL particles, forming foam cells. Such a lesion with foam cells, activated inflammatory cells is called as fatty streak.

Oxidised LDL, foam cells, the activated macrophages, T-cells produce various cytokines IL-1, TNF-α.. Under the influence of these cytokines, endothelium, macrophages and Tcells produce PDGF, FGF[21]. PDGF stimulates smooth muscle cell migration and proliferation. FGF stimulate the vascular smooth muscle cell to produce collagen and the various components of extracellular matrix together they form the fibrous cap. TNF- α induces apoptosis of foam cells causing exocytosis of its lipid content, which forms the lipid core. Such a lesion with lipid core, surrounded by activated T-cells, macrophages, platelets (occasionally), lined by a fibrous cap is called as a stable atherosclerotic plaque. Thus oxidised LDL is not only toxic to the endothelium and the surrounding cells in the intima but also chemotactic for monocytes and can activate monocyte derived macrophages to produce growth factors and cytokines. Hence it may be the principal culprit in advancing the lesions of atherosclerosis.

If the injury to the endothelium were a self-limited event and if endothelial function were restored, the proliferative lesions might be capable

of regressing. If this were the case, the lesions would be reversible and, if they had not reached a critical size, would be clinically silent. On the other hand, if the injury at focal sites in the artery wall is either of long standing or chronically repeated over period of many years, the lesion could continue to progress, becoming increasingly complex in terms of their composition. Because, the atherosclerotic plaque does not only have smooth muscle cells but also macrophages, which are capable of producing metalloproteinases and TNFα, both of which cause necrosis and digestion of the fibrous cap, this loss of fibrous cap is responsible for the complications of atherosclerosis, namely plaque rupture. This plaque rupture exposes the subendothelial extracellular matrix to the factors of coagulation in the circulation initiating the intrinsic pathway of coagulation – this is responsible for atherothrombosis.

**FACTORS INFLUENCING ATHEROGENESIS**

**UNMODIFIABLE RISK FACTORS**

1. Age
2. Male sex
3. Family History

**MODIFIABLE RISK FACTORS**

1. Cigarette smoking
2. Alcoholism
3. Insulin resistance & hyperglycemia
4. Hypertension
5. Obesity
6. Oxidative stress

7. Abnormal lipid profile

a. High total and LDL cholesterol

b. Low HDL cholesterol

8. High triglycerides

9. Lipoprotein(a)

10. Physical inactivity

**Age**

Age is a dominant influence .Death rates from ischemic heart disease rise with each decade even into advanced age, atherosclerosis is not clinically evident until middle age or later, when the arterial lesions precipitate organ injury. Between ages of 45 and 60 the incidence of myocardial infarction increases fivefold. Age related changes in cardiovascular system include diastolic dysfunction, degenerative changes in the conduction system, reduced responses to catecholamine and sympathetic stimuli. The oxidative stress associated with ageing and the co morbidity i.e., the appearance of the other risk factors are often cited as a reason for the high rates of CAD associated mortality and complications.

**Male sex**

The relationship of gender to the development and prognosis of atherosclerotic coronary heart disease is complicated[22]. Decades of observational studies have verified excess coronary risk in men compared with premenopausal women. At least part of the apparent protection against CAD in women derives relatively from their relatively higher HDL values compared to men .After menopause, HDL values fall in concert with

increased coronary risk. Women tend to develop atherosclerotic coronary heart disease approximately 10 years later than men.

This gender dependent differential risk is attributed to the protective function exerted by estrogens. Recently, a study of estrogens and their effect on smooth muscle cells and the other elements of atherogenesis showed that estrogens have an antiproliferative effect on smooth muscle cells and can be protective to the endothelium in relation to stimulation by growth factors, cytokines and other agents. Oestrogen is not only anti proliferative for smooth muscle but also has been shown to be capable of modulating acetylcholine mediated dilation of atherosclerotic coronary arteries[23].

**Family history of Early- Onset CHD**

Over 35 case-control and prospective studies have consistently identified an association between CHD and a history of first degree relatives with early onset CHD[73]. This risk generally persists even after adjustment for other risk factors. The family history most predictive of coronary disease is that of a first degree relative developing CHD at an early age. Although CHD in a male relative with onset at age 55 or less or a female relative with onset at age 65 or less is defined as a positive family history, the larger the number of relatives with early onset of CHD or the younger the age of CHD onset in the relative, the stronger the predictive value [74,75].

**Socioeconomic status**

A consistent relationship has been established between lower socioeconomic status and atherosclerosis. There has been the perception that conventional risk factors cluster in lower socioeconomic groups and that this phenomenon

can explain the increased incidence of atherosclerotic coronary heart disease[24.] However only 50% of atherosclerotic coronary heart disease can be explained by known risk factors. The socioeconomic status proved to be independent predictors in patients with established atherosclerotic coronary heart disease[25]. Although no simple relationship between socioeconomic status, risk for cardiovascular disease and long term outcome for manifest atherosclerotic coronary heart disease can be devised, the evident is consistent and persuasive that lower socioeconomic status is an independent and significant determinant of long-term outcome.

**Hypertension**

Several major prospective epidemiological studies have found that both systolic and diastolic hypertension have a strong, positive and graded relationship to CHD without evidence level of a threshold risk level of blood pressure[26-27]. The risk imposed by hypertension is heightened substantially when other risk factors are present. Hypertension clusters with insulin resistance, hyperinsulinemia, glucose intolerance, dyslipidemia, left ventricular hypertrophy and obesity and occurs in isolation in fewer than 20% of individuals[28]

The potential mechanisms by which hypertension may cause impaired endothelial function include increased endothelial permeability to lipoproteins, increased adherence of leukocytes, increased oxidative stress, and hemodynamic stress that may trigger acute plaque rupture, all these mediated by activation of NF-kB pathway and inactivation of eNOS enzyme.

## Hyperglycemia

Hyperglycemia is an independent risk factor for CHD, increasing the risk by two to three times for men and three to five times for women [29]. CHD is the leading cause of death in diabetic patients and approximately 25% of MI survivals have diabetes [30.] The CHD risk for a premenopausal diabetic woman is similar to the risk of a nondiabetic man, hence diabetes abolishes the protective effect of being a premenopausal female [31]. Diabetic women have twice the risk of recurrent MI compared with diabetic men [32]. The greater risk of CHD in diabetic women compared to diabetic men may be explained in part by the greater adverse effect of diabetes on lipoproteins in women [33].

Potential mechanisms by which hyperglycemia may cause atherosclerosis include impaired endothelial function, glycation of LDL, enhanced lipoprotein oxidation, increased fibrinogen, increased platelet aggregation, increased PAI-1, impaired fibrinolysis, increased small LDL. All these are attributed to the increased flux of glucose into glycolysis (glucose uptake and hexokinase activity in endothelium is insulin independent), as a result there is an increased NADH/NAD ratio, causing increased flux of electrons through electron transport chain, producing superoxide radicals. This cause DNA damage and the resultant ADP ribosylation of proteins inhibit glyceraldehyde 3 phosphate dehydrogenase of glycolysis, causing accumulation of glyceraldehyde 3 phosphate and its precursor fructose 6 phosphate. The former causes activation of protein kinase C pathway through DAG – protein kinase C pathway stimulates the production of various

cytokines and thereby stimulates inflammation. Fructose 6 phosphate stimulates hexosamine pathway, thereby stimulate N- glycosylation of many proteins like eNOS and inhibition of them, this causes NOS uncoupling and the resultant oxidative stress further aggravates the condition. Furthermore, there is increased formation of advanced glycation end product, which on binding to receptor for advanced glycation end products is found to stimulate NFkB pathway, causing all the features of atherosclerosis.

**Insulin resistance and hyperinsulinemia**

Resistance to insulin and compensatory hyperinsulinemia are the common metabolic basis of cluster of coronary risk factors, particularly hypertension, diabetes, hypertriglyceridemia, low HDL, predominance of small LDL, and an increased plasminogen activator inhibitor concentration [35, 36]. Hyperinsulinemia may rise blood pressure through sympathetic nerve stimulation and/or renal sodium retention. Insulin sensitivity is associated with endothelial nitric oxide production in healthy persons providing a clue as to how insulin resistance may promote CHD directly[37]. Furthermore, hyperinsulinemia has been found in a prospective study to be an independent risk factor for CHD in nondiabetic men after adjusting for body weight, blood pressure and dyslipidemia[38].

**Physical inactivity**

Physical inactivity roughly doubles the risk of CHD. Moderate intensity exercise reduces coronary atherosclerosis and widens coronary arteries in monkeys fed on atherogenic diet compared with monkeys fed the same diet but forced to be sedentary[39]. Physical activity slows progression of

angiographically defined coronary atherosclerosis in human[40]. Over 50 observational studies, primarily of men, have established that physical fitness, on the job of physical activity, and leisure time physical activity reduce the risk of CHD[41]. Higher levels of physical fitness and leisure time physical activity are associated with lower rates of mortality, independent of other risk factors[41]. The risk of MI and sudden cardiac death is greatest during exercise, leading some to question the benefits of exercise [42]. The overall risk of MI and sudden cardiac death, is however low among those who exercise regularly. The greatest reduction in risk is between sedentary individuals and those who do regular moderate intensity activity.

In addition to decreasing myocardial oxygen demand and increasing myocardial efficiency and electrical stability, other potential mechanisms by which physical activity may reduce CHD risk include increasing HDL, reducing blood pressure, reducing obesity, improving insulin sensitivity, decreasing platelet aggregation and increasing fibrinolysis[41] .

**Obesity**

Obesity promotes insulin resistance, hyperinsulinemia, hypertriglyceridemia, low HDL cholesterol, and LVH [43, 44]. Many observational studies have found that obesity strongly and positively correlates with the risk of CHD in univariate analysis. In multivariate analysis, when controlling statistically for risk factors such as hypertension, diabetes, and dyslipidemia, obesity is not found to be an independent risk factor. Rather it reflects that much of the adverse consequences of obesity are mediated through resultant metabolic risk factors acting as pathological links in the causal pathway. Nevertheless,

some large prospective observational studies of long duration indicate that obesity is an independent risk factor for coronary and cardiovascular mortality in men and women [45-47]. In general, the greater the degree of overweight, the higher the risk of coronary mortality. The central distribution of body fat predicts CHD in men independently of body-mass index and other major risk factors [48]. Weight loss improves insulin sensitivity and glucose disposal; reduces blood pressure, triglycerides and LVH; and increases HDL cholesterol [43-44].

**Oxidative stress**

Excessive production of reactive oxygen species has been implicated to play an important role in a number of cardiovascular pathologies, including hypertension, atherosclerosis, myocardial infarction, ischemia/reperfusion injury. ROS are generated in vascular cells by NADPH oxidases, uncoupled eNOS, and other enzymatic sources or as a product of mitochondrial respiration[49]. If this production goes unbalanced, it leads to exacerbation of pathophysiological processes. Superoxide radicals are found to cause oxidative modification of LDL. OxLDL, by activating NFkB pathway of inflammation is found to mediate the increased production of IL-1, increased expression of ICAM, both of which mediate the rolling of inflammatory cells. Furthermore there is increased production of PDGF and FGF, both of which cause smooth muscle cell proliferation and migration. This cycle is reinforced by the decreased production of NO, because the superoxide cause nitrosylation of eNOS and thereby it inhibits the enzyme activity. The decreased NO causes increased vascular reactivity and the resultant shear

stress will further stimulate NFkB pathway. Thus oxidised LDL is hypothesized to play a major role in the initiation and progression of atherosclerosis [50,51,52,53].

**Cigarette smoking**

Strong dose relationships between cigarette smoking and coronary heart disease have been observed in both sexes. Cigarette smoking increases risk two to threefold and interact with other risk factors to multiply risk. Pathophysiological studies have identified panoply of mechanisms through which cigarette smoking may cause CHD. Smokers have increased levels of oxidation products, including oxidised LDL. Cigarette smoking also lowers the cardioprotective levels of high density lipoproteins (HDL). These effects, along with direct effects of carbon monoxide and nicotine, produce endothelial damage. Possibly, through these mechanisms, smokers have increased vascular reactivity[54]. Cigarette smoking is also related to increased levels of fibrinogen[55] and increased platelet aggregability[56]. Thus cigarette smoking paves way for atherosclerosis by inducing oxidative stress and by altering coagulability.

**DYSLIPIDEMIA**

**Total cholesterol and LDL cholesterol**

Numerous prospective studies have identified a continuous, graded and direct relationship between serum cholesterol and CHD incidence [57]. The level of total and LDL cholesterol interacts with other risk factors to multiply risk [58]. Elevated LDL cholesterol levels have been related to recurrent events and CHD death in patients with established CHD [59]. Elevated LDL cholesterol

levels appear to be involved with all stages of atherogenesis – endothelial dysfunction, plaque formation and growth, and plaque instability and disruption. Elevated cholesterol levels in the plasma lead to an increased retention of LDL particles in the arterial wall, their oxidation and the secretion of various inflammatory mediators and chemoattractants [60]. LDL is also a potent mitogen for smooth muscle cells; progressive growth of atherosclerotic plaques can be halted by lowering of LDL cholesterol levels. Atherosclerotic plaques with a large lipid core and numerous lipid filled macrophages are prone to rupture [61]. Thus the epidemiological evidence strongly supports LDL-cholesterol's role in atherosclerosis.

Furthermore, small dense LDL are felt to be more atherogenic [62]. Possible explanation for this is that when a person has more of small LDL particles, for a given cholesterol content, the number of LDL particles will be more, and an LDL receptor can accept only one LDL particle at a time and hence the rate of metabolism of LDL is decreased, causing accumulation of LDL in the plasma. The second reason for the same is the endothelium will be more permeable to small LDL particle when compared to a normal LDL.

**Triglycerides**

The relationship between triglycerides and CHD has been less clear. In men, univariate analysis have consistently demonstrated a direct dose-response relationship. This relationship usually disappears after adjustment for other risk factors such as HDL cholesterol, obesity, and diabetes [63]. Hypertriglyceridemia however has been found to be an independent risk factor in women [64]. Several mechanisms have been proposed to explain the

triglyceride- CHD association. First, some patients with hypertriglyceridemia have a predominance of small, dense LDL particles. Second, fasting hypertriglyceridemia may be a marker of exaggerated postprandial hyperlipidemia, which may promote the uptake of atherogenic triglyceride rich lipoprotein remnants by endothelial cells[65]. Finally, serum triglyceride levels are strongly related to fibrinogen and factor VII in numerous epidemiological studies[66]. Therefore, a number of mechanisms, direct and indirect link serum triglycerides and CHD.

**Low HDL cholesterol**

Numerous prospective epidemiological studies have demonstrated a continuous, inverse relationship between HDL cholesterol levels and the incidence of CHD [67]. The total cholesterol to HDL cholesterol ratio is a better predictor of CHD than the HDL cholesterol level alone [67]. Two important mechanisms by which HDL is thought to play a protective role against atherosclerosis are reverse cholesterol transport and inhibition of LDL oxidation.

# ANGIOTENSIN-CONVERTING ENZYME (ACE)

Angiotensin-converting enzyme (ACE) is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells. Angiotensinogen is converted to angiotensin I by stimulation of renin. ACE then converts angiotensin I to angiotensin II, the main active product of the renin–angiotensin–aldosterone system (RAAS). The human ACE gene is located on chromosome 17q23, and includes 26 exons. The coding sequence codes for a 1306 amino acid protein, including a single peptide. The gene product, ACE, is composed of 2 homologous domains with 2 active sites. The ACE gene product plays an important role in cardiovascular homeostasis as ACE converts angiotensin I to angiotensin II, and is involved in the degradation of bradykinin. Bradykinin acts as a potent stimulator of nitric oxide (NO) release. NO plays a crucial role in protecting the endothelium from injury. Furthermore, it has been reported that hypertensive effects are mediated in a bradykinin-dependent manner. The ACE gene contains a polymorphism in the form of either insertion (I) or deletion (D) of a 287 base pair Alu repetitive sequence in intron 16. This polymorphism is shown to be associated with the interpersonal variability of ACE levels in circulating blood. The deletion allele at this gene site is associated with increased plasma ACE activity. Conflicting results have been reported regarding the association of ACE polymorphism with coronary artery disease (CAD).To date, there have been no data from South India to determine any association of ACE polymorphism with CAD or myocardial infarction (MI). In the present study, an attempt has been made to address the question whether

ACE polymorphism has any positive association with CAD patients from the South Indian state of Chennai. Available epidemiologic studies on Indians show a rise in the prevalence of CAD in urban Indians. Conventional risk factors for CAD do not completely account for the observed increase in premature CAD in people from the Indian subcontinent or in Asian Indians who have immigrated to the USA and Europe. As the incidence of CAD is increasing in India, especially in the younger population, it is important to identify the risk factors for CAD.

## STRUCTURE OF ACE

There are two forms of ACE in humans, encoded by a single gene located on chromosome 17 at q23; it is 21 kb in length and contains 26 exons and 25 introns. The longer form, known as somatic ACE (sACE), is transcribed from exons 1-12 and 14-26, whereas the shorter form, known as germinal or testicular ACE (gACE), is transcribed from exons 13-26. The promoter for s ACE is in the 5' flanking region of the first exon, whereas that for g ACE is located within intron 12 [132]. Somatic ACE consists of an intracellular domain, a transmembrane domain and two similar extracellular domains, the amino or N domain and the carboxy or C domain. The structure of the ACE gene is the result of gene duplication; the N and C domains are similar in sequence, and the homologous exons encoding the N and C domains (exons 4-11 and 17-24, respectively) are very similar in size and have similar codon phases at exon-intron boundaries. Each of the domains contains a catalytically active site characterized by a consensus zinc-binding motif (HEXXH in the single-letter amino-acid code, where X is any amino

acid) and a glutamine nearer the carboxyl terminus that also binds zinc; ACE and its homologs therefore make up the M2 gluzincin family [133].

The X-ray structure of testicular ACE, and its complex with the widely used ACE inhibitor lisinopril, at 2.0 Å resolution has been elucidated. This structure was determined using the anomalous scattering of the bound Zn atom at beam line **BM14** of the ESRF.The three-dimensional structure reveales that ACE is composed of a-helices for the most part, and incorporates a zinc ion and two chloride ions (Figure 33). In fact it bears little resemblance to carboxypeptidase A except in the active site zinc-binding motif. Instead, it resembles rat neurolysin and *Pyrococcus furiosus* carboxypeptidase, despite sharing little amino-acid sequence similarity with these two proteins. This similarity extends to the active site, which consists of a deep, narrow channel that divides the molecule into two subdomains. On top of the molecule is an amino-terminal 'lid', which seems to allow only small peptide substrates (2530 amino acids) access to the active site cleft this accounts for the inability of ACE to hydrolyse large, folded substrates.

**The Genes of the RAS:**

The genes encoding components of the renin-angiotensin system (RAS) present attractive candidates for cardiovascular disease. The RAS gene system comprises the renin, angiotensinogen (AGT), angiotensin I-converting enzyme (ACE), and angiotensin II receptor types 1 and 2 ($AGTR_1$, $AGTR_2$) genes. The renin gene maps to chromosome 1q32, spans approximately 12 kb, and comprises 10 exons and nine introns.[68, 69] The angiotensinogen gene maps to chromosome 1q42–43, spans approximately

13 kb, and comprises five exons and four introns[70, 71] ; exons 1 and 5 encode for the 5' and 3' untranslated regions of mRNA, respectively. The ACE gene maps to chromosome 17q23, spans 21 kb, and comprises 26 exons and 25 introns.[72, 73] The two major species of ACE mRNA are a 4.3-kb endothelial-type mRNA (transcription encompassing exons 1 to 26, excluding exon 13) and a 3-kb testicular type ACE mRNA (transcription encompassing exons 13 to 26). Exon 26 encodes for the functionally important membrane-anchoring domain of the ACE protein. The endothelial type of ACE mRNA is found not only in endothelial cells, but also in epithelial cells. The angiotensin II receptor type 1 gene maps to chromosome 3 and the angiotensin II receptor type 2 gene maps to chromosome X.[74,75]

Circulating RAS Components

RAS functions as an endocrine system. The renin gene is expressed primarily in the juxtaglomerular cells of the kidney, where renin is synthesized, stored, and released into the circulation. Prorenin is cleaved to form renin, which is stored in tissue granules until it is released in response to specific secretagogues. Secretion of renin from the kidneys is controlled by several factors. The macula densa are a specialized group of distal convoluted tubular cells that act as chemoreceptors for sodium and chloride levels in the distal tubule. Sodium retention increases blood volume, which is followed by an increase in blood pressure. This increase in blood pressure activates a negative feedback regulation of the juxtaglomerular cells in the kidney, which sense renal perfusion pressure and renin production are

inhibited. Renin secretion is autonomically modulated via sympathetic innervation of the renal tubules and arterioles.

Circulating renin catalyzes the angiotensinogen-to-angiotensin I conversion. The angiotensinogen gene is expressed in the liver, the site of AGT synthesis and release into the circulation. The angiotensin I (Ang I) generated by renin activity is a vasoinactive decapeptide. Conversion of angiotensin I to angiotensin II (Ang II) is the key reaction in the RAS pathway, generating the effector of the system, Ang II, a potent vasoconstrictor. The reaction is catalyzed by ACE (kininase II; EC 3.4.15.1), a zinc metallopeptidase member of the Alu family that functions as a dipeptidyl carboxypeptidase (DCP1). The mechanisms controlling the circulating ACE levels are less clear than those for renin. The most likely genetic control is at the level of transcription and would involve linkage disequilibrium with regulatory elements of the ACE gene. Once the protein is translated and bound to the cell membrane, release would require cleavage of the hydrophobic bonds that anchor the protein to the membrane. ACE cleaves the C-terminal His-Leu dipeptide from Ang I, generating the vasoactive octapeptide Ang II.[76]Further conversion of Ang II to Ang III is possible by cleavage of the aspartic acid from position 1 of the octapeptide; however, the generated Ang III is less potent as a vasoconstrictor, compared to Ang II.[76]Circulating ACE is found in biological fluids, such as plasma, amniotic and seminal fluids, and originates from endothelial cells.

ACE also acts as a protease on bradykinin, cleaving the C-terminal Phe-Arg dipeptide, with the net effect of inactivating this vasodilator.

Therefore, ACE enzymatic activity will result in a double effect: activation of a vasoconstrictor/pressor (Ang II) agent and inactivation of a vasodilator agent (bradykinin). Ang II is also an aldosterone-stimulating peptide. Aldosterone promotes depletion of potassium while promoting the retention of sodium and water; therefore Ang II exerts a negative feedback on rennin production due to volume expansion and/or to a direct effect on juxtaglomerular cells.

Tissue RAS Components

RAS also functions as a paracrine system. Ang II is demonstrated to be produced in multiple target organs by local RAS pathways. All components of the RAS, for example, are present in cardiac tissue[77, 78] ; transcripts for all RAS components are found in both atrial and ventricular tissue.[79, 80] However, under normal conditions the renin responsible for local/cardiac Ang I generation appears to derive from circulation, being of renal origin. Under pathological conditions, renin can be also produced in the heart.[81] The key component of the tissue RAS, as in circulating RAS, is ACE. At the cellular level, the ACE molecule projects into the extracellular space and is anchored to the plasma membrane by the C-terminal hydrophobic region that spans the membrane and ends in a short cytoplasmic tail. Ang II generated by ACE activity exerts its effects by binding to angiotensin II receptors, type 1 and type 2; $AGTR_1$ is the major mediator of physiological effects of Ang II (vasoconstriction, hypertrophy,catecholamine liberation at sympathetic nerve endings). Both $AGTR_1$ and $AGTR_2$ are transmembrane receptors, comprising seven membrane-spanning domains, and are coupled to

G-proteins. Both $AGTR_1$ and $AGTR_2$ mRNAs are expressed in the heart. However, $AGTR_1$ is the principal receptor mediating Ang II cardiac and circulatory effects. Cardiac effects include direct inotropic activity resulting in increased myocardial contraction, as well as cell growth and proliferation, resulting in cardiac remodelling, hypertrophy, and ventricular dilatation.[76] $AGTR_2$ appears to be the dominant receptor in both atrial and ventricular myocardium, as well as in the adrenal medulla and uterus.[76, 82] Functionally, $AGTR_2$ is an antagonist of $AGTR_1$, in as much as it has an antiproliferative effect.[83]

Ang II can also be generated in the tissues, including myocardium, by pathways other than RAS; non-RAS pathways involve nonspecific carboxypeptidases and chymotrypsin-like proteinases. An example of one of these is chymase (serine-proteinase), which catalyzes an efficient Ang II generation at tissue levels. Production of Ang II by these non-RAS alternative pathways is not inhibited by therapy with ACE inhibitors. The chymase pathway has been demonstrated in various cell types, including myocardium, endothelial cells, and mast cells.[84, 85] Chymase levels have been found higher in the ventricles than in the atria, and ventricle levels do not appear to change significantly in heart failure.[85]

The ACE gene polymorphism was first reported by Rigat et al in a study that addressed the role of the ACE gene in the genetic control of plasma ACE levels.[86] Normally, plasma ACE levels show marked interindividual variation but appear to be remarkably stable when measured repeatedly in the same subject. The normal ranges for plasma ACE levels and

the units of measurement depend on the detection method used. Rigat et al used direct radioimmunoassay measurement of the enzyme (in µg/L); subsequently, functional assays using spectrophotometric measurements (in U/L) havebeen used. Reference ranges for each method must be established in the testing laboratory. A current and widely used method is a spectrophotometric method using the synthetic tripeptide substrate *N*-[3-(2-furyl)acryloyl]-L-phenyl-alanylglycylglycine (FAPGG). The normal ranges are age-dependent and vary widely in adults (8–52 U/L).A previous study of healthy families had shown intrafamilial similarities of ACE levels, suggesting they are controlled by a major gene.[87] The polymorphism discovered by Rigat et al is of the insertion/deletion type; the two ACE alleles differ in size because of the insertion of a 287-bp DNA sequence in intron 16 of the ACE gene.[86] The ACE polymorphism was initially detected by restriction fragment length polymorphism (RFLP) analysis and Southern hybridization with a human ACE cDNA probe.[86] Subsequent studies of ACE polymorphism and disease associations used polymerase chain reaction (PCR) for genomic DNA amplification. The first PCR-based detection of the I/D ACE polymorphism was reported by Rigat et al,[88] who used a set of primers flanking the insertion sequence; the generated amplicons corresponding to the I and D alleles differ in size by the length of insertion sequence (ie, 287 bp) and allow discrimination between the three genotypes: II, ID, and DD.

Effect of Genotype on Enzymatic Levels

The ACE DD genotype is associated with increased circulating ACE levels, which are generally two times as high as those found for II genotypes; ID heterozygotes are associated with intermediate ACE levels.[98] This relationship of D allele dose and enzymatic levels, originally reported by Rigat et al, was repeatedly confirmed by other studies, for both circulating and cellular ACE.[99],[100,101,102]However, because the ACE I/D polymorphism is intronic, the mechanism of ACE overexpression in subjects with DD genotype is unclear. It is thought to be in linkage disequilibrium with a functional mutation in the gene. [140,141]

**Ang II, superoxide anions, and Inflammation**

Reactive oxygen species (ROS), generated by the membrane-bound NADPH-oxidase system[94] and stimulated by Ang II via its AT1-receptor, seem to be a pivotal step for atherosclerosis ; it is possible that this relationship is the result of tight linkage to another locus involved in the regulation of ACE gene expression.[107] DD genotype associated with increased ACE activity results in increased Ang I I production and thereby it contributes to atherosclerosis. Griendling et al. first demonstrated that Ang II stimulates the generation of ROS in vascular cells and macrophages, which are known activators for cytoplasmic signaling cascades such as nuclear factor-kB (NF-kB), mitogen-activated protein (MAP) kinases, or the Janus tyrosine kinases (JAK)/signal transducers and activators of transcription (STAT) cascade [90,91].Together, these mechanisms may enhance oxidative stress within the vascular wall and lead to the activation of redox-sensitive genes, such as those for proinflammatory cytokines[92]. These observations suggest that Ang

II may, via redox-sensitive mechanisms, activate IL-6 synthesis and release. Moreover, recent observations indicate that proinflammatory eicosanoids, such as leukotriene B4 or thromboxanes, are involved in AT1-receptor-dependent NADPH-oxidase activation. This latter pathway does not only link inflammation with the RAS but also links it with prothrombotic mechanisms. In this regard, proinflammatory eicosanoids are elevated in patients with unstable angina and play a critical role based on its vasoconstrictive and mitogenic potencies. When blocking the Ang II-induced effects by chronic ACE inhibition, macrophage recruitment into the vessel wall can be abolished in a rabbit model of atherosclerosis and apoE-deficient mice[103,104]. Moreover, blockade of the AT1 receptor by losartan prevents the accumulation of oxidative reactants in the atherosclerotic vessel wall and reduces the quantity of atherosclerotic lesion in an apoE-deficient animal model and monkeys[103,104]. Thus the interaction between ROS, inflammatory cells, and the RAS seemed to be important not only for the development of acute coronary syndrome, but also for the progression of atherosclerosis . With regard to the development of atherosclerotic lesions, evidence from other animal models, including rodents and primates, shows that ACE inhibition may reduce the extent of vascular lesions[103-105]. Additional mechanisms by which the RAS via Ang II may enhance the development of atherosclerosis involve the activation of thrombosis pathways via PAI-1 [95,96,97] or the stimulation of pro-inflammatory cytokines. Diet et al.[106] first demonstrated that Ang II–forming protease ACE is expressed in human atherosclerotic plaques. The authors demonstrated that in early- and

intermediate-stage atherosclerotic lesions, ACE was predominantly expressed in lipid-laden macrophages (similarly to pro-inflammatory cytokines) whereas in advanced lesions ACE was predominantly localized throughout the plaques microvasculature[105]. Potter et al. further demonstrated that lipid-laden macrophages contain Ang II in a primate model of atherosclerosis. In humans, at least two major enzymes, ACE and chymase, are involved in the conversion of Ang I to Ang II and may contribute to Ang II formation in coronary arteries. Further investigations demonstrated that in normal and atheromatous coronary artery segments of patients dying of noncardiovascular diseases that only ACE, but not chymase, was co localized with Ang II in the intima of stable atherosclerotic plaques. These findings suggest that ACE is apparently the primary source of Ang II in atherosclerotic human coronary arteries. Interestingly, Hoshida et al. demonstrated that tissue ACE activity is selectively upregulated in patients with ACS but serum-ACE activity is not. These observations suggest that tissue-ACE activity may represent an important regulator of Ang II formation at the atherosclerotic lesion.

## OTHER DISEASES ASSOCIATED WITH ACE GENE POLYMORPHISMS

### ACE Polymorphism and Venous Thrombosis

The DD genotype was found as a potent risk factor for thrombosis in patients undergoing total hip arthroplasty.[108] In a case-controlled study, Philipp et al investigated the association of ACE polymorphism, with

postoperative venous thrombosis. The plasma ACE levels in this study showed the same pattern previously reported by others, with the highest values in DD patients, intermediate values in ID heterozygotes, and the lowest values in II patients.[108] Another study that showed an association between the DD genotype and an increased risk of venous thrombosis was reported by Dilley et al[109] for an African-American population, with a threefold relative risk in men but not in women.

ACE Polymorphism and Nephropathy

Because of its central role in RAS, the ACE polymorphism has been extensively investigated, again with conflicting results in renal disease, as recently reviewed by Schmidt and Ritz.[110] There is increasing evidence that the progression of diabetic nephropathy is more rapid in patients with DD genotype.[112, 110, 111]The ACE genotype appears to predict the therapeutic efficacy of ACE inhibition of proteinuria; DD genotype patients are resistant to this renoprotective therapy, whereas ID and II genotype patients have a significant reduction in the degree of proteinuria.[113, 114, 115,116.]

ACE Polymorphism and Coronary Restenosis after Stent Implantation

Because RAS has been implicated in the development of neointimal hyperplasia,[117] ACE activity is a crucial step for the RAS pathway, and the resulting increased generation of Ang II is a potent growth factor for smooth muscle cells,[118]the hypothesis has been advanced that genetic factors affecting RAS and particularly ACE gene expression may be important in pathogenesis of coronary restenosis after stenting. Indirect experimental evidence was obtained by demonstrating that ACE inhibitors block

neointimal thickening after arterial balloon denudation in rats, guinea pigs, and rabbits.[119, 120]The clinical relation between restenosis after coronary stenting and ACE polymorphism was investigated by Amant et al[121] ; the association of the number of D alleles and poststent restenosis was independent of other risk factors.[121] The increased ACE activity due to the presence of the D allele, mainly in the homozygous state, may account for the higher degree of coronary neointimal thickening found in these patients.[121]

## ACE Polymorphism and Hypertension

The association between ACE polymorphism and essential hypertension is controversial. A significant association of the ACE gene D allele with essential hypertension was documented in the African-American (Duru *et al*., 1994),Chinese (Chiang *et al*., 1996), and Japanese populations(Morise *et al*., 1994; Nakano *et al*., 1998). On the other hand,the I allele was associated with high blood pressure in an Australian population with strong evidence of familial hypertension (Zee *et al.,* 1992). It has been suggested that the population heterogeneity in the association of ACE I/D polymorphism with essential hypertension may be due to significant variations of population backgrounds (Barley *et al*.,1994). Response to ACE inhibitors in hypertensive patients appears to be determined at least in part by the ACE genotype in the study of Ohmichi et al,[126].

## ACE polymorphism and breast cancer;

Evidence from animal models has suggested that angiotensin II stimulates neovascularisation by promoting arteriolar smooth muscle cell proliferation, and this has led to increased interest in the role that angiotensin II may play a role in promoting angiogenesis in neoplastic growth. In addition, angiotensin II may act as a mitotic factor by inducing or regulating gene expression in cell cycle progression, and angiotensin II receptor blockade effectively reduced transforming growth factor ß1-dependent tumor progression *in vivo* . Captopril, a prototype ACE inhibitor, has been shown to inhibit proliferation in a variety of cell types, including human breast cancer cells [126,127,128,129,] and to reduce tumour growth in experimental models of cancer [130,131]

Ace polymorphism was also studied in other diseases like cardiomyopathy, stroke, Alzheimer's disease, Systemic Sclerosis.

# AIM OF THE STUDY

Coronary Artery Disease causes more deaths and disability and incurs greater economic costs than any other illness in the developed and developing world. A high-fat and energy-rich diet, smoking, and a sedentary lifestyle are associated with the emergence of CAD. These classical risk factors account for only 50% of the causes of heart disease. It is unknown in 50% of the cases. Inherited lipoprotein disorders account for remainder of the causes. Genetic factors play an important role in susceptibility to these disorders. The knowledge of genetic factors of coronary artery disease may help in explaining the molecular basis of this disorder and in designing prevention and treatment methods. Literature evidences point to the role of ACE gene I /D polymorphism in the causation of atherosclerosis.Eventhough ACE gene polymorphism is located in intron 16 of ACE gene, it is thought to be in linkage disequilibrium with another functional mutation in the gene. The differences in the degree of linkage disequilibrium between this quantitative trait locus and I/D polymorphism are cited as a reason for the differences in the associations of the polymorphisms with CAD in different populations.

The aim of the study is

1. To compare the ACE gene I/D polymorphism between cases and controls.

2. To compare the lipid profile values between cases and controls

# MATERIALS AND METHODS

## STUDY POPULATION

### CASES

The study sample comprised 61 unrelated South Indian Coronary Artery Disease patients (56 male, 5 female) of mean age 55.1years . Inclusion criteria was more than 50 % stenosis of at least one of the major coronary arteries. Patients with less obstruction were excluded .All patients with acute myocardial infarction or unstable angina, and patients with ischemic or idiopathic cardiomyopathy were also excluded.

### CONTROL SUBJECTS

Totally 62 controls subjects (55 male, 7 female) of mean age 55.5 years were studied and the controls were recruited from outpatient department during their visit for non cardiac cases. Age, sex and other confounding factors like diabetes, hypertension were matched. For all diabetic controls, tread mill test was done .Only those with negative tread mill test were included in the study.

### METHODS

Recumbent blood pressure and 12 lead ECG were recorded on each subject after a thirty minute rest on the couch. Height and weight were recorded and 3 mL of blood was collected by venipuncture after fortnight fasting in EDTA test tube. EDTA tube was centrifuged at 2000 rpm for twenty

minutes to get the buffy coat for DNA extraction and the plasma was utilised for lipid profile estimation.

## BUFFY COAT SEPARATION

Buffy coat was separated by centrifugation of EDTA tubes at 2000 revolutions per minute for 20 minutes. Buffy coat was transferred to 2mL eppendorf and was used for DNA extraction. Plasma separated was used for lipid profile estimation.

## BIOCHEMICAL MARKERS

Total cholesterol (TC), high density lipoprotein cholesterol (HDL-c) and triglyceride concentration (TGL) were determined enzymatically using kits and XL-300 auto analyzer at Centralized Biochemistry Laboratory at G.G.H, Chennai-3. Low density lipoprotein cholesterol (LDL-c) was calculated using Friedwald's formula.

## DNA EXTRACTION BY MODIFIED HIGH SALT METHOD[162]

### RBC Lysis:

- 400µL of buffy coat in a 2mL eppendorf is mixed with 1.6mL of 0.17M ammonium chloride and mixed by inversion until red cells are lysed for about 10 minutes
- The cells are centrifuged at 4000rpm for 10minutes.

- The white cell pellet is washed with 800µL of 0.17M ammonium chloride solution. The procedure is repeated till a clear white cell pellet is obtained.

**WBC Lysis**

- To the pellet 500 µL of TKM I solution is added. It is centrifuged at 10,000rpm for 10minutes.

**Nuclear Lysis**

- Discard the supernatant. To the pellet add 500 µL of TKM II solution. To that add 300 µL of 6M Nacl and 50 µL of 10% SDS.
- Mix well (vortex), Centrifuge at 10,000 rpm for 10 minutes.
- Save the supernatant. Transfer it to 1.5mL eppendorf.

**DNA Precipitation**

- To the supernatant double the volume of 100% ethanol is added.
- The sample is stored at -20◦C for 1 hour.
- Then it is centrifuged at 10,000 rpm for 20minutes at 4◦C in a refrigerated centrifuge.

- The supernatant is discarded. To this 500 µL of 70% ethanol is added. The pellet is mixed and centrifuged at 10,000 rpm for 10minutes at 4◦C.

- Supernatant is discarded and the pellet is air dried.

**Storage**

- To the pellet 30 µL of LTE buffer is added and the extracted DNA is stored at -20◦C for future use.

**Identification**

- Extracted DNA was identified by 0.8% agarose gel electrophoresis with a constant voltage of 7V/ cm and comparison with a known molecular weight 1kb DNA ladder.Figure:1

**POLYMERASE CHAIN REACTION**

- ACE gene was amplified using,

    - Forward primer – 5'-CTGGAGACCACTCCCATCCTTTCT–3' and
    - Reverse primer – 5'-GATGTGGCCATCAATTCGTCAGAT – 3'

**Primer Reconstitution**

Primers are supplied in lyophilized form. Autoclaved distilled water is used to prepare 100 × concentrations i.e. 10times the molecular weight of

primer is the volume of water required to prepare 100 × concentrations which is 100μmolar solution.

- From this stock solution 10 × concentration is prepared as the working solution for PCR.

**MASTER MIX:**

- Genei Red Dye master mix in the following composition was used.

- Master Mix consists of a unique inert red dye in addition to basic components necessary for PCR.

    - Reaction buffer consisted of Tris Hcl -10mM at pH 8.3 KCl - 50mM
    - MgCl2 - 1.5mM acts as catalyst.
    - dNTP's were used in a concentration of 2.5mM each.
    - Taq polymerase in a concentration of 1.5 U.

- Primers were used in a concentration of 5 pmol and DNA was used in a concentration of 200ng.

- PCR was carried out in a reaction volume of 25 μL with the following components;

    - PCR master mix– 12.5 μL
    - Forward primer – 0.9 μL
    - Reverse primer – 0.9 μL

- DNA – 2 .0µL
- Distilled water – 8.7 µL
- Total – 25 µL

- Amplification was carried out in an Mc Genei thermal cycler with the following cycling conditions.
  - Initial denaturation – $94^0$ C -5min
  - 30 cycles of
    - Denaturation – $94^0$C – 1 min
    - Annealing - $58^0$C – 1min
    - Extension - $72^0$C – 1min
  - Final extension at $72^0$C - 10 min.

- Amplified products 490 bp PCR for I allele and 190bp PCR product for D allele was identified by agarose gel electrophoresis by comparison with a known 100bp DNA ladder. Thus, each DNA sample revealed one of three possible patterns after electrophoresis: a 490 bp band (II genotype), a 190 bp band (DD genotype), or both 490 and 190 bp bands (I/D genotype) Figure 2.

**AGAROSE GEL ELECTROPHORESIS**

- PCR product is run on agarose gel in a 50 mL agarose cast as follows: 1g of agarose is weighed and dissolved in 50mL of TAE buffer with a pH of 8.0.

- It is microwaved for 60 secs, cooled and 2.5 µL of ethidium bromide (10mg/mL) is added. It is poured into a cast and allowed to solidify for 15 min before it is kept in the electrophoresis tank. 8 µL of PCR product is loaded onto wells and 4 µL of 100bp DNA ladder is loaded onto single well as a marker. It is electrophoresed at 8V/cm for 45min and visualized under UV illumination.

# LIPID PROFILE

The biochemical parameters undertaken for the study were determined using the following methodologies:

**Estimation of Plasma Total Cholesterol**

**Method**

Cholesterol Esterase – Cholesterol Oxidase

**Kit used**

Autospan of Span Diagnostics Ltd.

**Principle**

$$\text{Cholesterol Ester} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4- Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red quinine} + 4H_2O$$

The concentration of cholesterol in the sample is directly proportional to the intensity of the red complex (red quinone), which is measured at 500nm.

**REAGENTS**

**Reagent 1 (Enzymes / Chromogen)**

Cholesterol Esterase ≥ 200U/L

Cholesterol Oxidase ≥ 250U/L

Peroxidase ≥ 1000 U/L

4- Aminoantipyrine 0.5 mmol/L

**Reagent 1A (Buffer)**

Pipes buffer, pH 6.90 50mmol/L

Phenol 25mmol/L

Sodium Cholate 0.5 mmol/L

**Standard (Cholesterol 200mg/dL)**

Cholesterol 2g/L

**Procedure**

To 1 mL of the reconstituted reagent, 10 µL of plasma is added and reading is taken after 5 mins of incubation at 37° C.

**Reference Values**

Cholesterol : 150-260 mg /dL

**ESTIMATION OF PLASMA TRIGLYCERIDE**

**Method**

Enzymatic Colorimetric method

**Kit Used**

Autopak of Bayer Diagnostics

**Principle**

Lipoprotein Lipase

Triglyceride + $H_2O$ → Glycerol + Fatty acid

Glycerol Kinase

Glycerol + ATP → Glycerol -3- Phosphate + ADP

Glycerol -3- Phosphate Oxidase

Glycerol -3- Phosphate → Dihydroxy acetone phosphate + $H_2O_2$

Peroxidase

2 $H_2O_2$ + 4 Aminoantipyrine + ADPS → Red Quinone + $H_2O$

The intensity of purple colored complex formed during the reaction is directly proportional to the triglyceride concentration in the sample and is measured at 546nm.

**REAGENTS**

**Reagent 1 (Enzymes / Chromogen )**

Lipoprotein Lipase ≥ 1100U/L

Glycerol Kinase ≥ 800U/L

Glycerol -3- Phosphate Oxidase ≥ 5000 U/L

| | |
|---|---|
| Peroxidase | ≥ 350 U/L |
| 4- Aminoantipyrine | 0.7 mmol/L |
| ATP | 0.3 mmol/L |

**Reagent 1A (Buffer)**

| | |
|---|---|
| Pipes buffer. pH | 7.50 50mmol/L |
| ADPS | 1mmol/L |
| Magnesium salt | 15 mmol/L |

**Standard (Triglycerides 200mg / dL)**

Glycerol (Trig. Equivalent) 2g/L

**Procedure**

To 1 mL of the reconstituted reagent 10 µL of plasma is added and read at 546nm after incubation at 37°C for 5mins.

**Reference Range**

| | |
|---|---|
| Males | 60- 165 mg/dL |
| Females | 40- 140 mg/dL |

**Estimation of HDL Cholesterol**

Method Immunoinhibition

Kit used - Erba XL System Packs

**Principle**

Chylomicrons, VLDL, and LDL fractions in plasma are separated from HDL by immunoinhibition. Anti human β-lipoprotein antibody in reagent 1 binds to lipoproteins (Chylomicrons, VLDL, and LDL) other than HDL. The antigen-antibody complexes formed block enzyme reactions when reagent 2 is added. Cholesterol esterase and cholesterol oxidase in reagent 2 react only with HDL-C. Hydrogen peroxide produced by enzyme reactions with HDL-C, yields a blue coloured complex upon oxidase condensation with F-DAOS and 4-aminoantipyrine (4-AA) in the presence of peroxidase(POD) . The intensity of the blue color complex formed at 593 nm is proportional to the HDL-C in the sample.

Anti-Lipoprotein B antibody

LDL, VLDL & Chylomicrons antigen antibody complex

CHE & CO

HDL-C + $H_2O$ +$O_2$ 4-cholestenone + Fatty acid + $H_2O_2$

Peroxidase

$H_2O_2$ + DAOS + 4AAP Blue colored complex + 2$H_2O$

**REAGENTS**

**Reagent 1**

Goods buffer pH 7.0 30.0mmol/L

4-AAP 0.9mmol/L

POD 2400U/L

Ascorbate oxidase 2700U/L

Antihuman β lipoprotein antibody

**Reagent 2**

Goods buffer, pH – 7.0 30.0mmol/L

CHE 4000U/L

CO 20000U/L

F-DAOS 0.8mmol/L

**Calibrator**

HDL-C 56.5mg/dL

**Procedure**

Reagent 1 & 2 are mixed in the ratio of 3:1 or 1 bottle of reagent 1 is mixed with 1 bottle of reagent 2 and placed in the auto analyser with the following assay parameters:

Assay type : 2 point

Primary wavelength nm : 600, Secondary wavelength nm : 700

R-1 volume : 270, R-2 volume : 90

Reaction direction : increasing, Sample volume : 3 µL

Calibration : straight

**Reference Values**

Adult male : 35.3 – 79.5 mg /dL

Adult female : 42.0 – 88.0 mg / dL

**VLDL and LDL Cholesterol**

These parameters were calculated using Friedwald's formula given below:

LDL-C = TC - (HDL-C+ VLDL-C)

VLDL-C = TGL/5

# STATISTICAL ANALYSIS

1. Allele frequencies were calculated by allele counting.

2. Age, BMI, serum lipid levels were compared between control subjects and patients by students t test.

3. Genotype frequency distribution between cases and controls were compared with a $\chi^2$ test for 2*2 contingency table.

4. Logistic regression analysis was performed to evaluate the interaction between ACE genotypes II/DD/ID and other variables in relation to the prevalence of Coronary Artery Disease. Independent variables included in the analysis were age (quantitative), sex (male/female), smoking(yes/no), Alcoholism(Yes/No), Hypertension (Yes/No), Diabetes (Yes/No), Serum Levels of Cholesterol, Triglycerides (Quantitative). The analysis was executed by SAS Statistical program Version 6.10 for Macintosh.

# RESULTS

Table 1 shows Age, Sex, BMI, High Density Lipoprotein levels and conventional risk factor distribution among patients and control subjects. Since all the confounding factors were matched, there were no significant differences between the two groups. There was a significant difference in the High Density Lipoprotein level – low in cases (37.2+8), high in controls (51.5 +12.5) .LDL is high in cases than controls but not significant, this could be because they were on treatment with cholesterol lowering medications.

Table 2.1 and 2.2 shows Genotype distribution and Allele frequencies of human ACE gene in patients with CAD and control subjects. ACE genotype distribution was in agreement with the Hardy-Weinberg expectations.

- DD genotype was more frequent among cases (65.7 %) when compared to controls (39.3%). In contrast ID and II was more common among controls (61.7%) when compared to cases(34.3%). There was a significant difference in the distribution of II genotype also between cases and controls .P value is 0.001.

- There is also significant difference between the cases and controls in the distribution of D and I alleles

Table 3 shows the Genotype distribution of ACE gene between I+ and I- genotypes. I+ genotype is common among controls (61.7%) compared with cases (34.3%)

Table 4 shows the age- and sex-adjusted odds ratio between I+ allele (ID+II genotypes) and I-(DD genotype), was 3. (95% CI, 1.4 to 6.7; P=.01).This shows that I allele protects against atherosclerosis and homozygous DD genotype favors atherosclerosis.

Table 5 shows the multiple logistic regression analysis and shows that no significant difference between cases and controls when age,sex, diabetes, hypertension smoking and alcoholism are compared showing that all the confounding variables are perfectly matched between cases and controls and there is significant difference in HDL levels (p value .000) and ace genotype(p value 0.045) between cases and controls proving our hypothesis.

# DISCUSSION

Besides very well known risk factors, genetic factors also play a role in the development of coronary artery disease. Genetic factors differ in various populations. Among these, ACE gene polymorphism has most frequently been studied and proposed as a coronary artery disease risk factor. ACE gene Polymorphism determines the serum and tissue ACE activity which is high in subjects with DD genotype (Malik et al., 1997)[134]. ACE by causing high angiotensin II (Ang II) and low bradykinin levels may increase the risk of CAD (Cambien et al., 1992)[135]. Angiotensin II increases the macrophage derived growth factor and platelet derived growth factor which have a role in the genesis of atherosclerosis (Keidal et al., 1993)[136]. Furthermore, Ang II leads to LDL- C oxidation and stimulates neutrophil, macrophage and T-lymphocytes (Farber et al., 1990[137]; Keidal et al., 1993). ACE decreases nitric oxide release via the bradykinin-kallikrein system and causes endothelial dysfunction which has also an important role in the genesis of atherosclerosis. Homozygous deletion subset of the ACE I/D polymorphism is associated with deteriorated endothelial function (Mulder et al., 2003)[138]. It has been demonstrated by various studies that the ACE D allele is associated with the risk of CAD in various populations. However, other studies show that ACE gene polymorphism is not associated with CAD and MI. A large case–control study by Gardemann et al[139]. on Caucasian samples has shown that the D allele was associated with CAD in patients <61.7 years of age, while Wesolowska et al. studied French Canadian

subjects, and found that ACE polymorphism is not associated with premature CAD.

Hence, the clinical relevance of ACE gene polymorphism still remains unclear. As CAD is a multifactorial disease, the ACE gene alone may not have a direct effect on the severity of CAD and premature death. However, the homozygous DD genotype is found to be significantly increased in our study samples.

In this study we have not confirmed the DD genotypes using insertion specific primers as previous studies showed that there is possibility of mistyping[89] due to preferential amplification of D allele and all the DD genotype positive cases to be confirmed by using another insertion specific primer. This is the drawback of our study. And we have not tested the phenotypic variation associated with ACE genotypes, one more limitation of our study. The present study is important as there is a need for a robust confirmation of the risk genes for CAD, even if the effect is small, so as to contribute to our understanding of the pathology of CAD, and determine potential therapeutic strategies.

# CONCLUSION

Cardiovascular disease is the major cause of morbidity and mortality. It is well known that the etiology of this devastating disorder involves both genetic and environmental factors. Sequence variants of the components of the rennin angiotensin-aldosterone system and the kallikrein-kinin system are suggested to have significant influences on cardiovascular homeostasis. Thus, the ACE gene has been recognized as a top candidate gene for cardiovascular research. ACE gene polymorphism is involved in the pathogenesis of coronary artery disease via the plasma and tissue ACE levels. CAD is a preventable disease and elimination is postulated before the end of the 21st century .Elucidation of genetic modifiers is a prerequisite to genetic screening and comprehensive prevention of Coronary Artery Disease and this is the basis of our present study.

In our study homozygous ACE DD genotype was more frequent in CAD patients compared to ID/II. The presence of even single I allele protects against atherosclerosis. Conclusively ACE DD genotype is significantly associated with coronary atherosclerosis. This association needs to be confirmed in a large group of population.

# SCOPE FOR FURTHER STUDY

1. ACE gene polymorphism along with circulating and tissue ACE activity, angiotensin II levels, can be done in the future to know the pathogenesis behind the disease.

2. ACE gene therapy can be tried in those with DD genotype. CAD,being a multifactorial disease this does not look promising.

3. Pharmacogenetic studies on ACE gene I/D polymorphism will help in understanding individual responses to treatment and in personalizing treatment regarding type and dose of antihypertensive medicine. Those with DD genotype can be given angiotensin receptor blockers instead of ACE inhibitors.

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